

## Antioxidants and Pigments of *Aspergillus niger*

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Extracts of six fungi, four yeasts, and two species of *Streptomyces* were tested for antioxidant activity when added to lard. Extracts of *Aspergillus niger*, the microorganism that showed the strongest antioxidant activity, were subjected to adsorption and gel permeation chromatography. Two fractions that protected lard against oxidation were obtained by chromatography of *A. niger* extract on Sephadex LH-20 and Bio-Beads S-X2. One of these fractions contained a gummy brown pigment BR, the other a bright yellow crystalline pigment Y. Pigment BR showed strong carbonyl group and methyl-methylene group absorption in the infrared. Apparently, on the basis of spectral data, pigment Y has a linear naphthopyrone structure. Pigment BR was obtained consistently from *A. niger* mycelium, while pigment Y was formed sporadically. Results indicated that more than one substance in *A. niger* mycelium have antioxidant properties and that brown and yellow pigments apparently are associated with antioxidant activity. Synergistic effects may be important in the strong antioxidant activity of *A. niger* extracts.

### 1. Introduction

Previous studies have shown antioxidant activity in extracts of microorganisms.<sup>1-4</sup> To our knowledge, the only compound with strong antioxidant properties identified in a microorganism is 2-(6-hydroxy-2-methoxy-3,4-methylenedioxyphenyl)-benzofuran<sup>5</sup> isolated from bakers and brewers yeast.<sup>2</sup> In a previous study, bacteria were shown to contain materials that protect lard against oxidation.<sup>6</sup> Further studies of antioxidant activity were carried out on six fungi, four species of yeast and two of *Streptomyces*. Of the microorganisms tested, *Aspergillus niger* possessed the strongest antioxidant activity. Hence, the active extracts of *A. niger* were examined to elucidate the nature of the antioxidant material.

Chromatographic studies of *A. niger* extract showed that yellow and brown pigments were present in fractions exhibiting antioxidant activity.

The yellow pigments of *A. niger* have been investigated by a number of workers.<sup>7-14</sup> Reviews of literature prior to 1950 may be found in Ref. 12 and 14. No reports of antioxidant activity related to pigments of *A. niger* have been found in the literature.

Several yellow pigments of *A. niger* have been isolated and their structures determined. These are: riboflavin,<sup>9</sup> flavasperone (asperxanthone),<sup>15</sup> aurasperone A,<sup>13,16</sup>

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aurasperone B,<sup>13,17</sup> aurasperone C,<sup>13,17</sup> asperenone (asperyellone),<sup>18,19</sup> and asper-rubrol.<sup>11</sup>

Zajic and Kuehn<sup>14</sup> also reported the presence of an acidic carotenoid pigment and a pterinoic pigment in some isolates of *A. niger*. A yellow carotenoid pigment is produced during conidiophore development induced by nitrogen limitation in cultures of *A. niger*, according to Anderson and Smith.<sup>20</sup> The same workers<sup>21</sup> reported that during conidiophore development induced by the addition of certain amino acids and tricarboxylic acid-cycle intermediates to a medium containing high levels of ammonium ions, a yellow pigment similar to asperyellone was produced. A variety of polyhydroxy anthraquinone pigments have been identified in different *Aspergillus* species;<sup>22</sup> however, this ring system has not been reported as yet for *A. niger*.

## 2. Experimental

### 2.1. Microorganisms and growth media

The *Streptomyces* were isolated from soil; the fungi and yeast were from the laboratory stock culture collection except Yeast YD which was isolated from dried bakers yeast and Yeast RY which was a red pigmented strain isolated from compressed yeast cake.

The media used for the production of cells were: *Medium A*: NH<sub>4</sub>Cl, 1.0 g; KH<sub>2</sub>PO<sub>4</sub>, 1.5 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.12 g; FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g; MnSO<sub>4</sub>·H<sub>2</sub>O, 0.01 g; Edamin S (Sheffield), 40.0 g; glucose, 2.5 g (25 ml of a 10% solution was sterilised separately); distilled water to make the volume 1000 ml. *Medium B*: Case peptone, 1.0 g; 1.0 M phosphate buffer, pH 7.0 (equimolar Na : K), 5 ml; glucose, 0.25 g (2.5 ml of a 10% solution sterilised separately); distilled water to make the volume 100 ml. *Medium C*: Case peptone, 1.0 g; 1.0 M phosphate buffer, pH 7.0 (equimolar Na : K), 5 ml; distilled water to make the volume 100 ml. *Medium D*: Difco<sup>a</sup> nutrient broth, 0.8 g; Difco yeast extract, 0.5 g; glucose, 1.0 g; distilled water to make the volume 100 ml. *Medium E*: Difco nutrient broth. The media (200 ml) were dispensed into 1 litre Erlenmeyer flasks and sterilised by autoclaving at 121 °C for 15 min before inoculation.

Two other media were also used for preparation of large amounts of *Aspergillus niger* mycelium: Difco tryptose phosphate broth and BBL trypticase soy broth. The inoculated media were incubated at 25 °C for 24 h (static), then shaken at 200 rev/min for 48 h.

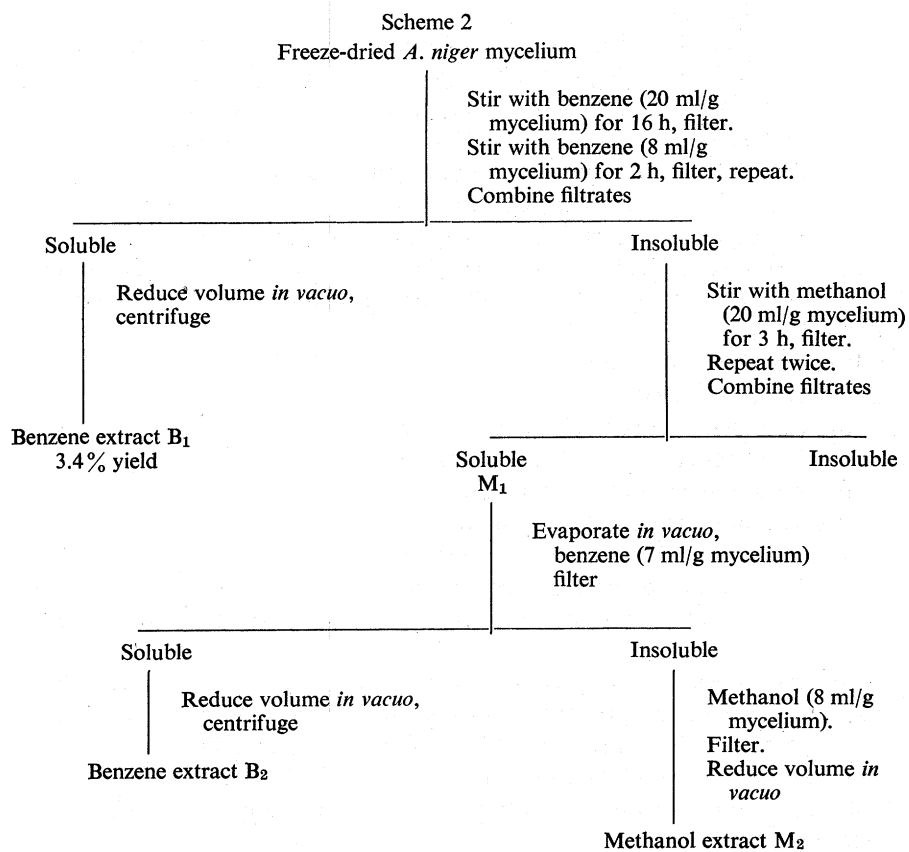
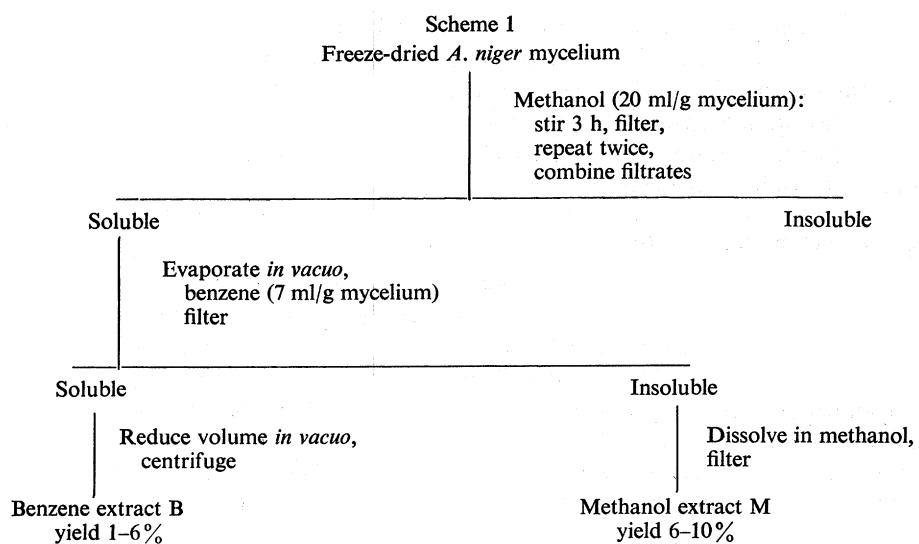
### 2.2. Preparation of cell extracts

Cell extracts of microorganisms were prepared as previously described.<sup>6</sup> Benzene extract B was prepared from freeze-dried *A. niger* according to Scheme 1 and benzene extracts B<sub>1</sub> and B<sub>2</sub> were prepared according to Scheme 2.

### 2.3. Assay for antioxidant activity

Extracts of various microorganisms were tested for antioxidant activity as described previously.<sup>6</sup> Peroxide values (PV) were determined by the iodometric method of Lea.<sup>23</sup>

<sup>a</sup> Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.



In experiments with *A. niger*, the extracts and chromatographic fractions were tested for antioxidant activity on the basis of a given weight of the original mycelium, e.g. a sample equivalent to 2 g wet wt or 0.25 g dry wt of mycelium was sufficient to exhibit antioxidant activity when added to 15 g of lard stored at 60 °C. A sample was considered to have antioxidant activity if it took 70 h or longer to reach a PV of 30 compared to 40 h for the control.

#### 2.4. Partition of extract with NaOH

Benzene extract B (25 ml), prepared from 22 g of mycelium, was extracted with two 10 ml portions of 2% NaOH solution. The benzene phase was then washed with 3 × 5 ml of water, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated *in vacuo* to give the NaOH-insoluble fraction. The aqueous phase (extracts and washings) was acidified with conc. HCl to pH 2 and extracted with 5 × 10 ml of benzene. The benzene phase was washed with 2 × 5 ml of water, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and concentrated *in vacuo* to give the NaOH-soluble fraction.

Extract B was treated with 2% NaHCO<sub>3</sub> and 1 N HCl solutions in a similar manner.

#### 2.5. Column chromatography

Sephadex LH-20 (Pharmacia, Uppsala, Sweden) was swollen in methanol and packed into a 1.5 × 43 cm column. Extract B, equivalent to 35 g mycelium (wet wt), in methanolic solution was applied to the gel and the column was eluted with 300 ml of methanol. Fractions (5 ml) were collected and their u.v. and visible spectra recorded. The effluent was pooled into fractions C–G on the basis of similarity of spectra.

Amberlite XAD-2 (30–50 mesh, Rohm and Haas Co., Philadelphia, Pa.) was washed exhaustively with methylene chloride and methanol to remove ultraviolet-absorbing impurities. Extract B, equivalent to 10.7 g of freeze-dried mycelium, in 15 ml of methanol was applied to the resin, 2.9 × 24 cm column, packed in methanol. The column was eluted with methanol to yield fractions R (100 ml), S (200 ml), and T (200 ml). The column then was eluted with 500 ml of methylene chloride (fraction U). Each fraction was evaporated *in vacuo* and dissolved in benzene.

Bio-Beads S-X2 (200–400 mesh, Bio Rad Labs, Richmond, Cal.) was purified by washing with methylene chloride and methanol, then swollen in benzene and packed into a 1.5 × 42 cm column. Benzene extract B from 4–15 g of freeze-dried mycelium was applied to the column and eluted with 100 ml of benzene. Five ml fractions were collected at a flow rate of 0.5 ml/min and were pooled into fractions 1–8 on the basis of colour. The first 25 ml of effluent were discarded. The column had a pale yellow colour which could not be removed by additional elution with benzene. Residual material was readily removed from the column with 50 ml of methanol to yield fraction 9.

Silica gel (Woelm, activity I, Waters Associates, Inc., Framingham, Mass.) was used either as the dry adsorbent or suspended in benzene to give a column 1.8 × 20 cm. *A. niger* extract B, equivalent to 5–10 g of freeze-dried mycelium was applied to the column and eluted with 70 ml of benzene, 50 ml of 4% methanol in benzene, 50 ml of 20% methanol in benzene, and finally 70 ml of methanol. Ten fractions of eluted material were collected.

Similar experiments were carried out using columns of silica gel (Merck EM Reagent, activity 2-3, Brinkmann Instruments, Inc., Westbury, NY) and aluminum oxide (Camag, chromatographic grade, basic, A. H. Thomas Co., Philadelphia, Pa.).

All of the fractions obtained by column chromatography were examined by thin-layer chromatography and tested for antioxidant activity.

## **2.6. Thin-layer chromatography**

Thin-layer chromatography was carried out on precoated layers: Merck Silica Gel F 254 on aluminum (Brinkmann Instruments, Inc., Westbury, NY); Eastman Chromagram sheet 6061, silica gel without fluorescent indicator (Eastman Kodak, Rochester, NY); Quanta Gram PQ5-500 silica gel plates (Bodman Chemicals, Narberth, Pa.).

### **2.6.1. Solvent systems**

1. Benzene.
2. Benzene-ethyl acetate-ethanol (96 : 2 : 2 by vol.).
3. Chloroform-acetic acid (9 : 1 by vol.).
4. i-Propanol-ammonia-water (8 : 1 : 1 by vol.).

### **2.6.2. Visualisation of components**

Ultraviolet light, iodine vapour, phosphoric acid (Stahl no. 208), 5% molybdophosphoric acid (Stahl no. 168), 2,6-dichloroquinonechloroimide (Stahl no. 66).

## **2.7. Spectra**

Ultraviolet and visible spectra were recorded on Bausch and Lomb Spectronic 505 and Cary 14 Spectrophotometers. Ultraviolet spectra were determined for solutions in methanol and visible spectra for solutions in methanol or benzene. Infrared spectra were recorded on a Beckman IR-8 Spectrophotometer.

## **2.8. Solvent purification**

Reagent grade solvents were freed from prooxidants prior to use for extraction and column chromatography. Methanol was purified by the method of Lund and Bjerrum.<sup>24</sup> Benzene was purified as described by Hornstein and Crowe<sup>25</sup> or by passage through a column of aluminum oxide (Woelm, basic, Waters Associates, Inc., Framingham, Mass.). Methylene chloride was used without further purification.

# **3. Results and discussion**

## **3.1. The presence of antioxidant material in various microorganisms**

The microorganisms studied, the conditions used for their production, and the antioxidant activity of the microbial extracts added to lard are shown in Table 1. Antioxidant activity, when present, was found in the benzene-soluble fraction, as was the case with bacterial extracts.<sup>6</sup> Fractions that protected lard for at least 20 days at 50 °C were considered to have strong antioxidant activity.

Extracts of fungi showed strong antioxidant activity with *Aspergillus niger* exhibiting the strongest antioxidant activity of the microorganisms examined in this or the previous study.

**Table 1.** Methods of production of microorganisms and the effect of their fractions<sup>a</sup> on oxidation of lard

Microorganism	Growth medium <sup>b</sup> and conditions	Number of days for oxidation of lard <sup>c</sup> at 50 °C	
		Benzene insoluble	Benzene soluble
<i>Fungi</i>			
<i>Aspergillus niger</i> no. 172	B <sup>d,f</sup>	11.7	54.0
<i>Thamnidium elegans</i> no. 195	B <sup>e,f</sup>	10.9	34.3
<i>Geotrichium candidum</i> no. 165	A <sup>e,g</sup>	10.8	29.2
<i>Mucor sufu</i> no. 251	B <sup>d,f</sup>	10.8	28.8
<i>Penicillium roqueforti</i> no 174.	B <sup>d,f</sup>	10.7	24.2
<i>Penicillium camemberti</i> no. 193	B <sup>d,f</sup>	12.5	23.4
<i>Streptomyces</i> :			
<i>Streptomyces</i> sp. no. A2	E <sup>d,h</sup>	10.7	14.7
<i>Streptomyces</i> sp. no. A4	E <sup>d,h</sup>	13.1	— <sup>j</sup>
<i>Yeasts</i> :			
<i>Candida lipolytica</i> no. 181	C <sup>e,i</sup>	11.3	23.6
Yeast RY	D <sup>d,i</sup>	10.2	20.9
Yeast YD	D <sup>d,i</sup>	10.6	18.2
<i>Hansenula saturnis</i> no. IU21	D <sup>d,i</sup>	11.3	16.1

<sup>a</sup> The cells were extracted with methanol and the methanol-soluble material was extracted with benzene. 100 mg of microbial fraction/15 g of lard.

<sup>b</sup> Composition of the growth media is described under Materials and Methods.

<sup>c</sup> Number of days for PV of lard to reach 15. The control sample of lard reached a PV of 15 in 10 days.

<sup>d</sup> 25 °C.

<sup>e</sup> 20 °C.

<sup>f</sup> 24 h static then shaken 48 h.

<sup>g</sup> 24 h static then shaken 72 h.

<sup>h</sup> 48 h static then shaken 48 h.

<sup>i</sup> 24 h shaken.

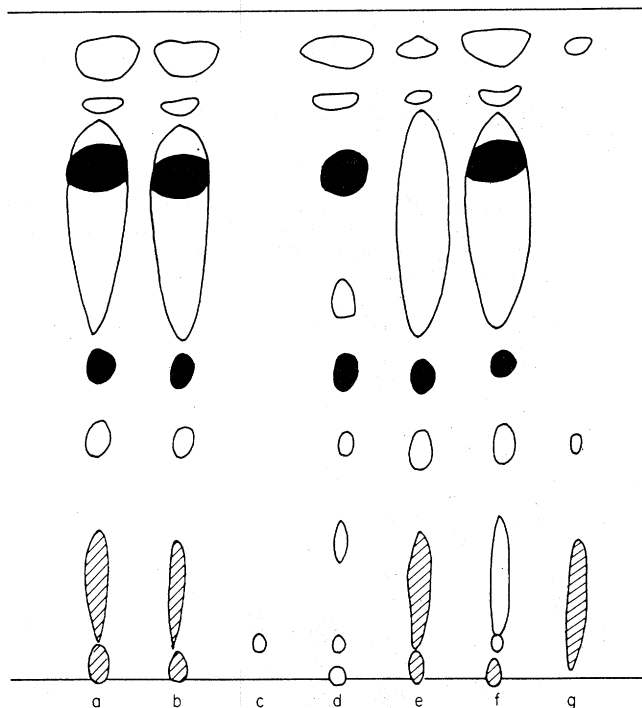
<sup>j</sup> Very little of the microbial material was soluble in benzene. The methanol-soluble fraction was assayed.

### 3.2. Antioxidant activity of *A. niger*

Extracts of *A. niger* were chromatographed on several types of columns to isolate the material possessing antioxidant properties.

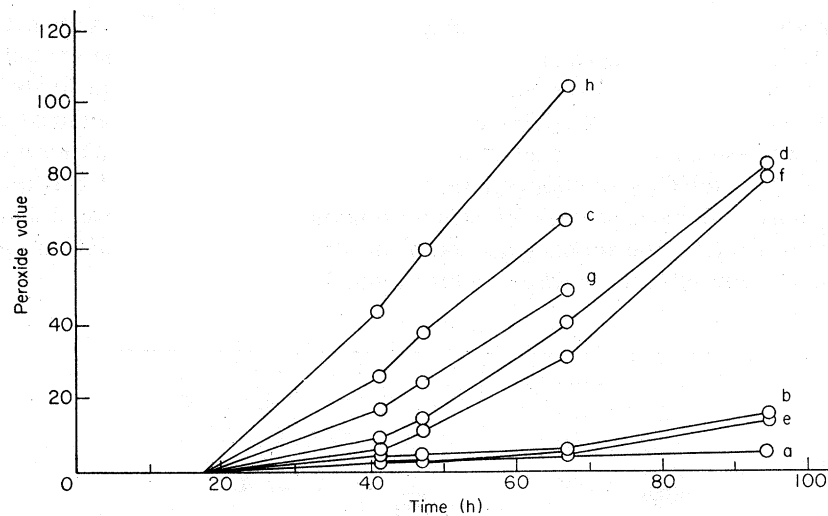
Gel chromatography of the benzene extract B on alkylated polydextran Sephadex LH-20 with methanol as eluent gave complete recovery of pigments from the column. These were partially separated into several brown and yellow bands. Most of the nonpigment material was eluted in the first two fractions together with some brown pigment. Ultraviolet and visible spectra of eluted fractions indicated the presence of material with  $\lambda_{\max}$  270–280 nm and 410–420 nm, apparently due to a number of closely related pigments. Ergosterol, a major component of the extract, was found in fraction D. Fraction F contained a bright yellow pigment. When added to lard, fractions D and F showed antioxidant activity. This indicated that more than one substance may be responsible for the antioxidant activity of *A. niger*.

In an attempt to separate groups of compounds, *A. niger* extract B was treated with 2% NaOH, 2% NaHCO<sub>3</sub>, and 1 N HCl solutions. In each case, the extract was separated into soluble and insoluble fractions. Ergosterol and neutral lipids were found in the NaOH-insoluble fraction. The NaOH-soluble fraction contained all of the pigments and showed antioxidant activity. Most of the *A. niger* extract was insoluble in 2% NaHCO<sub>3</sub> solution; however, pigments were distributed between the two fractions, both having some antioxidant properties. Since only a trace of material dissolved in 1 N HCl, the antioxidants and pigments were found in the HCl-insoluble fraction. These results are shown in Figures 1 and 2.

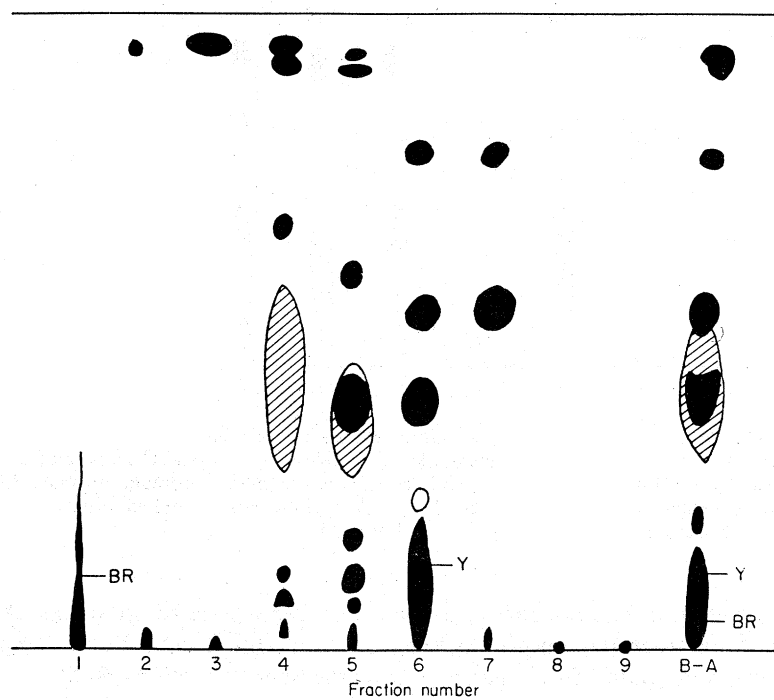


**Figure 1.** Thin-layer chromatogram of *A. niger* benzene extract B treated with acid and base. a, Benzene extract of *A. niger*; b, HCl-insoluble; c, HCl-soluble; d, NaOH-insoluble; e, NaOH-soluble; f, NaHCO<sub>3</sub>-insoluble; g, NaHCO<sub>3</sub>-soluble. Layer: Eastman Chromagram 6061, Silica gel. Solvent: benzene-ethyl acetate-ethanol (96 : 2 : 2). Detection: cross hatched areas, visible light (pigments); white areas, iodine; black areas, phosphoric acid.

Good separation of components of extract B in methanolic solution was obtained by column chromatography on polystyrene resin Amberlite XAD-2. Polar compounds and pigments were eluted from the resin with methanol (fractions R-T). Nonpolar compounds were then eluted with methylene chloride (fraction U). Only fraction S, which contained most of the yellow pigments, showed weak antioxidant activity. Prooxidant effects were noted in the other fractions.



**Figure 2.** Antioxidant activity of *A. niger* benzene extract B treated with acid and base. Sample equivalent to 8.6 g of *A. niger* mycelium (wet wt) was combined with 15 g of lard and stored at 60 °C. a, Benzene extract of *A. niger*; b, HCl-insoluble; c, HCl-soluble; d, NaOH-insoluble; e, NaOH-soluble; f, NaHCO<sub>3</sub>-insoluble; g, NaHCO<sub>3</sub>-soluble; h, control.



**Figure 3.** Thin-layer chromatogram of *A. niger* extract B-A separated on Bio-Beads S-X2 column. Layer: Merck Silica Gel F 254 on aluminium. Solvent: benzene-ethyl acetate-ethanol (96 : 2 : 2). Detection: molybdophosphoric acid. BR, brown pigment, Y, yellow pigment.

Although the antioxidant activity of individual batches of mycelium varied, one benzene extract (B-A), prepared according to Scheme 1, exhibited very significant activity. This extract was fractionated on a column of porous styrene-divinylbenzene copolymer Bio-Beads S-X2. Thin-layer chromatographic analysis of the fractions obtained is shown in Figure 3. Two fractions (1 and 6) possessed antioxidant activity. Fraction 1 contained mostly brown pigment (BR) while fraction 6, the more active fraction, contained yellow pigment (Y). A later experiment indicated that prolonged storage (3 months at 5 °C) decreased the antioxidant activity of extract B-A. Fraction 6 retained the yellow colour but not the antioxidant activity.

During chromatography of *A. niger* extract on Bio-Beads S-X2, an unknown component having a red-purple colour in benzene ( $\lambda_{\max}$  505, 535, and 570 nm) was separated in fractions 6–8.

Results of column chromatography on alumina and silica gel (activity 2–3) lend support to the idea that pigments may be involved in the antioxidant activity of *A. niger*. Pigments were not eluted from alumina columns with either benzene or methanol. Only small amounts of yellow pigments were eluted with methanol from silica gel (activity 2–3) columns. None of the eluted fractions showed antioxidant activity.

To simplify the extraction of antioxidant material from *A. niger*, the freeze-dried mycelium was extracted directly with benzene (Scheme 2) to yield fraction B<sub>1</sub>. The mycelial residue was then extracted with methanol to yield fraction M<sub>1</sub>. Both B<sub>1</sub> and M<sub>1</sub> showed antioxidant activity. Treatment of the material in M<sub>1</sub> with benzene yielded a benzene-soluble fraction B<sub>2</sub> and a benzene-insoluble fraction M<sub>2</sub>, both showing antioxidant activity. Fraction B<sub>1</sub> contained lipids, sterols, fluorescent compounds, and pigments. Thin-layer chromatographic analysis showed that small amounts of these compounds were also found in fraction B<sub>2</sub> because of difficulty in achieving complete extraction. B<sub>2</sub> contained additional pigments and more polar compounds.

The active extract B<sub>1</sub> was subjected to gel chromatography on Bio-Beads S-X2 with benzene as eluent and a fraction possessing antioxidant properties was obtained. It corresponded to fraction 6 obtained previously (Figure 3) and contained bright yellow pigment Y. No brown pigment or other material was eluted in fraction 1. Extract B<sub>2</sub> was also chromatographed on Bio-Beads S-X2 to yield fraction 1 which contained brown pigment BR and showed antioxidant activity.

The benzene-insoluble fraction M<sub>2</sub> contained large amounts of dark brown pigments, also mannitol, and other water-soluble components. This material has not been investigated further in the present work although moderate antioxidant activity was consistently found in this fraction.

Benzene extracts B<sub>2</sub> from different batches of *A. niger* mycelium consistently showed moderate antioxidant activity. However, benzene extracts B<sub>1</sub> showed large variations in activity as well as in the amount of yellow pigment.

Our experiments indicate that at least three different substances possessing antioxidant activity may be present in *A. niger* mycelium:

1. Material directly extractable from the mycelium with benzene. The antioxidant activity of this fraction may be associated with the presence of a bright yellow pigment Y, although this could not be confirmed at the present time.
2. Material soluble in, but not directly extractable with, benzene. This fraction can

be obtained from the mycelium by preliminary extraction with methanol. The active fraction consists mainly of a mixture of brown pigments BR.

3. Methanol soluble-benzene insoluble fraction consisting mainly of dark brown pigments. This fraction also contains mannitol and other water-soluble compounds.

At the present stage of our work, the antioxidant activity of *A. niger* extracts cannot be associated definitely with any particular compound. Several constituents of *A. niger* such as: mannitol,<sup>10</sup> citric acid,<sup>26</sup> ascorbic acid,<sup>27</sup> phosphatidyl choline,<sup>28</sup> phosphatidyl ethanolamine,<sup>28</sup> and ubiquinone<sup>29</sup> have been reported to show antioxidant activity toward fats. A review of the results of numerous investigations concerned with the antioxidant properties of the above-mentioned compounds show that these compounds have rather weak antioxidant properties when added alone to oils and fats but exhibit a synergistic effect when used in combination with other antioxidants.<sup>30,31</sup> The combined effect of all the weak antioxidants present in *A. niger* may result in strong antioxidant activity of the extract when added to lard, although separation of the material may not yield any fraction showing significant activity.

### 3.3. Pigments of *A. niger*

Our experiments indicate that the pigments of *A. niger* may play an important role in the antioxidant activity shown by the mycelial extracts. In all our experiments, only fractions containing pigments showed antioxidant activity when added to lard.

We did not attempt to duplicate any of the work on pigments of *A. niger* reported by various investigators since our primary concern was antioxidant activity. Several different, apparently closely related pigments, judging by their u.v. spectra, are present in small amounts in the benzene extract of *A. niger*. We examined primarily two pigment fractions (described above): brown pigment BR and yellow pigment Y.

Pigment Y (m.p. 190–193 °C) precipitated on standing from fraction 6 (Figure 3) obtained by chromatography of extract B-A on Bio-Beads S-X2 column. The i.r. spectrum of pigment Y (Figure 4) indicated bands characteristic of hydroxy and carbonyl groups ( $\nu_{\max}$  3400, 1640, 1580, and 1161  $\text{cm}^{-1}$ ). The u.v. spectrum (Figure 5) showed absorption at 228, 275, 319, 331, and 407 nm. The similarities of the i.r. and

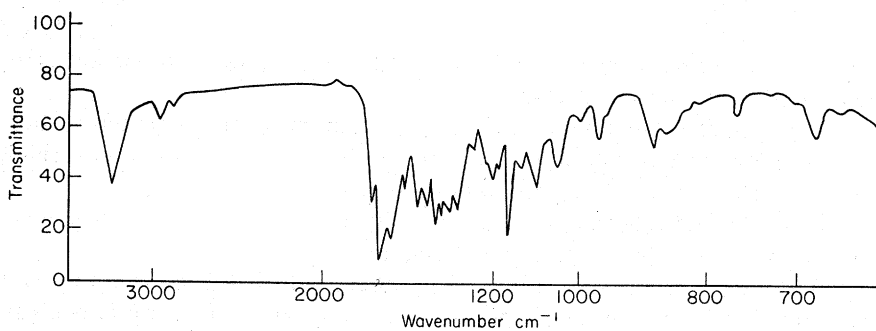


Figure 4. Infrared spectrum of yellow pigment Y from *A. niger*; KBr pellet.

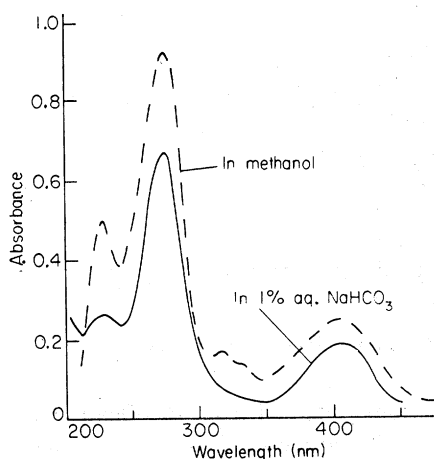


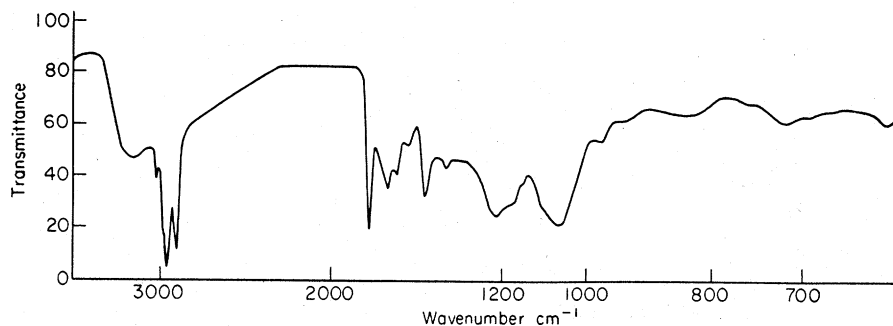
Figure 5. Ultraviolet spectrum of yellow pigment Y from *A. niger*.

u.v. spectra of pigment Y to those of the aurasperones isolated from *A. niger*<sup>13</sup> suggest that it may possess a linear naphthopyrone structure. The spectral properties of pigment Y are also similar to those of asperflavin<sup>32</sup> and flavomannin,<sup>33</sup> yellow pigments isolated from *Aspergillus flavus* and *Penicillium wortmanni*, respectively.

Thin-layer chromatographic experiments indicate that the yellow pigment may be a mixture of several closely related compounds. Also changes in the pigment may occur during thin-layer chromatography. Rapid browning of the yellow pigment was noted on some silica gel layers (Quanta Gram PQ5-500 plates and Merck Silica Gel F-254 sheets) resulting in poor recovery of sample, while better results were obtained on Eastman Chromagram 6061. Part of pigment Y may be converted to a new yellow pigment during thin-layer chromatography on silica gel. This was apparent from two-dimensional chromatography in *i*-propanol–ammonia–water (8 : 1 : 1 by vol.) since one of the three yellow spots obtained did not lie on the diagonal and showed only slight migration. Chromatography on cellulose layers with the same solvent system gave only two very diffuse yellow spots. The yellow pigments eluted from thin-layer chromatograms with methanol all had similar u.v. spectra.

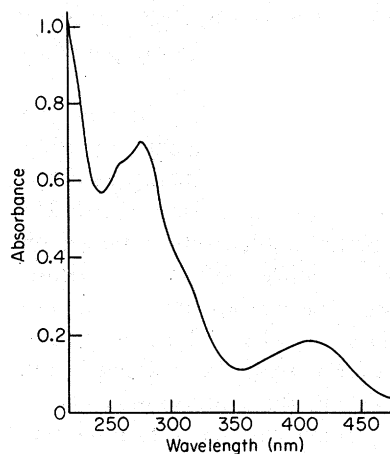
The brown pigment fraction BR, having antioxidant activity, was obtained by chromatography of *A. niger* extract B or B<sub>2</sub> on Bjo-Beads S-X2 column. The same pigment fraction also was obtained from an extract of 5 g of freeze-dried mycelium by elution with benzene from a 1.5 × 20 cm column of silica gel, Woelm, activity 1. The brown pigment was obtained in 10 ml of effluent after discarding the first 15 ml. The other components were then eluted from the column with mixtures of benzene and methanol.

The i.r. spectrum of pigment BR (Figure 6) showed strong methyl-methylene absorption ( $\nu_{\max}$  2915 and 2841 cm<sup>-1</sup>) and carbonyl absorption ( $\nu_{\max}$  1732 and 1214 cm<sup>-1</sup>). A strong broad band at 1057 cm<sup>-1</sup> may be due to the presence of an alcoholic hydroxy group. Bands in the 1000–625 cm<sup>-1</sup> region were very broad and weak. The band at 720 cm<sup>-1</sup> may be due to long chain CH<sub>2</sub> absorption. The i.r. spectra, as



**Figure 6.** Infrared spectrum of brown pigment BR from *A. niger*; film on NaCl plate.

films on NaCl plates, of the brown pigment fractions obtained from Bio-Beads S-X2 column and silica gel column were identical. The u.v. spectrum (Figure 7) of pigment BR showed absorption at 260 (sh), 278, and 410 nm. Unlike the yellow pigment Y which dissolved in 1%  $\text{NaHCO}_3$  solution, the brown pigment BR was insoluble in  $\text{NaHCO}_3$  but soluble in 2%  $\text{NaOH}$ .



**Figure 7.** Ultraviolet spectrum of brown pigment BR from *A. niger*; in methanol.

Additional work is needed to further elucidate the nature of the pigments produced by *A. niger* and their role in inhibiting the oxidation of fats.

#### Acknowledgements

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